

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 11/14, 9/20 // C01B 33/20		A1	(11) International Publication Number: WO 95/22606 (43) International Publication Date: 24 August 1995 (24.08.95)
(21) International Application Number: PCT/DK95/00076 (22) International Filing Date: 21 February 1995 (21.02.95) (30) Priority Data: 0207/94 21 February 1994 (21.02.94) DK		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): PEDERSEN, Sven [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). LARSEN, Anne, Mørkeberg [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). AASMUL, Per [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).			
(54) Title: METHOD FOR PRODUCTION OF AN IMMOBILIZED ENZYME PREPARATION AND USE OF THE IMMOBILIZED ENZYME PREPARATION			
(57) Abstract <p>The method comprises that a liquid enzyme composition and a particulate silica carrier is introduced into a granulator or extruder. The preparation is cheap and exhibits satisfactory properties during use in fat related processes, if the enzyme is a lipase.</p>			

NZAS-0027158

BEST AVAILABLE COPY

METHOD FOR PRODUCTION OF AN IMMOBILIZED ENZYME PREPARATION AND USE OF THE IMMOBILIZED ENZYME PREPARATION

The invention comprises a method for production of an immobilized enzyme preparation, comprising an enzyme applicable for organic synthesis in a mainly organic medium devoid of free water, and a use of the immobilized enzyme preparation. The most common enzyme which is applicable for organic synthesis in a mainly organic medium devoid of free water is lipase. Examples of other enzymes of this kind are proteases, amidases, esterases, oxidoreductases, and nitrilases. In the following the invention usually will be described with reference to lipase as the predominant example of an enzyme which is applicable for organic synthesis in a mainly organic medium devoid of free water. The term "organic synthesis" is to be understood as generally accepted in organic chemistry. Thus, typical examples of organic syntheses included in the scope of the invention are the following: reesterifications, transesterifications, interesterifications, acylations, epoxidations, aminolyses, ammoniolyses, oxidations, and reductions. The term "mainly organic medium devoid of free water" is to be understood as a one phase medium, the organic part of which amounts to at least 50% w/w.

Immobilized lipase preparations are used as catalysts for interesterification and other fat related processes, e.g. cocoa butter substitute production. In case of a batch reaction the catalyst has to be separated from the reaction mixture for reuse when the reaction is finished. Thus, a good filtrability of the catalyst is needed for satisfactory performance.

WO 90/05778 describes a method for production of an immobilized lipase preparation useable for e.g. margarine production. This preparation comprises a macroporous silica carrier.

EP 140 542 describes an immobilized lipase preparation for interesterification of fats. This preparation comprises an anion exchange resin carrier.

Both these prior art immobilized lipase preparations suffer from the disadvantage that they are expensive. Especially in relation to the production of margarine, which is produced in millions of tons per year on a global basis, it is important to minimize production costs.

Thus, the purpose of the invention is the provision of a method for production of a cheap immobilized enzyme preparation which should exhibit technical properties equal to or almost equal to the prior art immobilized enzyme preparations, especially in regard to filtrability after a finished batchwise margarine production and in regard to a low pressure drop in columns for continuous performance, in case the enzyme is a lipase, and of a use of such immobilized enzyme preparation.

The method according to the invention for production of an immobilized enzyme preparation comprising an enzyme applicable for organic synthesis in a mainly organic medium devoid of free water, is characterized by the fact that a liquid enzyme composition and a particulate silica carrier with a particle size below around 100 μm is used as materials to be introduced into a granulator or extruder, whereafter a granulation or an extrusion is carried out. The liquid enzyme composition can be non-aqueous, e.g. on alcoholic basis, or aqueous. The particulate silica carrier can exhibit a broad particle size distribution, e.g. between around 5 μm and 100 μm . In this specification with claims "silica" is to be understood as either silica or a silicate, e.g. magnesium silicate. It is to be understood that the invention both comprises the situation, where a particulate immobilized lipase composition with a particle size distribution similar to the particle size distribution of the particulate silica carrier is first produced, whereafter the granulation or extrusion is carried out (vide Examples 6 and 7), and the situation, where the production is carried out in one step only (vide Examples 1 to 5). Also, it is to be understood that the enzyme may act as a binder during the granulation or extrusion, and/or that a specific binding agent can be added, e.g. gelatin or polyvinylpyrrolidone. During the method according to the invention preferably an atomization has to be carried out, usually an atomization of the liquid enzyme composition and/or an atomization of the binding agent in liquid form. Also, it is to be understood that the apparatus used in the method according to the invention is of no special importance to the invention, inasmuch as any granulator, e.g. a fluid bed spray granulator, or any extruder can be used.

A powdered immobilized lipase preparation on silica basis is described, e.g. in WO 88/02775, page 11, lines 21-24. This immobilized lipase preparation is

completely unfit for both batchwise and continuous fat related processes, due to poor filtrability after a batch process and generation of a high pressure loss during a continuous column process.

Immobilized lipase preparations are described in EP 579928 and in 5 Appl. Microbiol. Biotechnol. (1988) 28:527-530, but none of these prior art lipase preparations comprise a silica carrier.

In Chem. Abstract Vol. 118 (1993): 55095v an immobilized lipase preparation on a silica carrier is described. However, the method according to the invention comprising particle size of the carrier and granulation or extrusion is not 10 described.

Surprisingly it has been found that the immobilized enzyme preparation prepared in accordance with the method according to the invention in the first place is dramatically cheaper than the comparable prior art immobilized enzyme preparations, and in the second place that it exhibits technical properties equal to 15 or almost equal to the prior art immobilized enzyme preparations, e.g. in regard to filtrability after a batchwise fat related process and generation of a low pressure loss during a continuous fat related process, if the enzyme is a lipase.

A preferred embodiment of the method according to the invention is characterized by the fact that the enzyme is a lipase.

20 A preferred embodiment of the method according to the invention is characterized by the fact that the lipase in the liquid lipase composition is a thermostable lipase.

A preferred embodiment of the method according to the invention is characterized by the fact that the lipase in the liquid lipase composition is produced 25 by cultivation of a microorganism containing a gene encoding for and expressing a lipase derived from a strain of *Humicola* species, *Candida antarctica* or *Rhizomucor miehei*.

A preferred embodiment of the method according to the invention is characterized by the fact that the proportion between the amount of the liquid lipase 30 composition and the weight of particulate silica carrier is at least 100,000 LU/g of carrier (dry weight). LU is the lipase activity unit defined in AF 95.1/2-GB, which can

be obtained on request from Novo Nordisk A/S. The LU assay uses tributyrin as a substrate for determination of the lipase activity.

A preferred embodiment of the method according to the invention is characterized by the fact that the silica has a purity of at least 50%, preferably at least 75%.

A preferred embodiment of the method according to the invention is characterized by the fact that a granulator is used, preferably a high speed mixer or a mixer granulator.

A preferred embodiment of the method according to the invention is characterized by the fact that a liquid composition of a binder, preferably gelatin or polyvinylpyrrolidone, is introduced by atomization into the granulator or extruder during the granulation or extrusion.

A preferred embodiment of the method according to the invention is characterized by the fact that the granulation or extrusion is carried out for production of the immobilized lipase preparation with a particle size distribution corresponding to an amount of at least 90% between 50 μm and 2,000 μm .

The use of the immobilized enzyme preparation prepared by means of the method according to the invention is for the process catalyzed by the enzyme.

The use of the immobilized lipase preparation prepared by means of the method according to the invention is for fat related processes. It is to be understood that such fat related processes can be performed batchwise or continuously. When performed batchwise it has been found that the immobilized lipase preparation produced by means of the method according to the invention exhibits a satisfactory filtrability when the enzymatic process has come to an end, and when performed continuously it has been found that the immobilized lipase preparation produced by means of the method according to the invention exhibits a good physical strength resulting in a satisfactory performance of the column.

A preferred embodiment of the use according to the invention is for interesterification of fats and is characterized by the fact that liquid fats or fatty mixtures, including free fatty acids or fatty acid esters, are contacted with the immobilized lipase preparation.

A preferred embodiment of the use according to the invention is for synthesis of glycerides or other fatty acid esters and is characterized by the fact that a mixture of glycerol or substituted glycerols or other types of alcohols and free fatty acids is contacted with the immobilized lipase preparation.

5 A preferred embodiment of the use according to the invention is for synthesis of glycolipids. The synthesis of glycolipids with immobilized lipase preparations in general is described in Björkling, F. et al. (1989), J. Chem. Soc., Chem. Commun., p. 934-935.

The invention will be illustrated by the following examples.

10 All manufacturing examples (1 - 8) illustrate the batchwise embodiment of the method according to the invention. For production in industrial scale ordinarily the continuous embodiment will be preferred. Example 9 is a use example.

15 The use according to the invention is illustrated indirectly in Examples 1 - 8, in consideration of the fact that every BAUN determination illustrates the use (interesterification) according to the invention. The use according to the invention is illustrated directly in Example 9.

Fig. 1 which is directly related to Example 9, shows the conversion in relation to an ester synthesis performed as a continuous column operation, depending on time.

20

EXAMPLES

EXAMPLE 1

65 g of a powder of synthetic magnesium silicate, Celkate T-21 (Manville) was introduced into a high speed mixer with an impeller which can be operated with a speed of 900 rpm. 75 g of *Humicola lanuginosa* lipase liquid 25 concentrate (prepared according to Danish Patent No. 157560, with *Humicola lanuginosa* DSM 3819, dry substance content 30%, with an activity of 700,000 LU/ml) was continuously atomized onto the silica powder over a period of approx. five minutes with running impeller. The formed granulate was dried overnight at room

temperature and sieved (300 - 700 μm). The moisture content was adjusted to 10% and the sample analyzed to 2.6 BAUN/g. The lipase activity assay expressed in BAUN (Batch Acidolysis Units Novo) measures the initial rate of incorporation of decanoic acid into high oleate sunflower oil (10% water, 70°C). A detailed description 5 of the method (MP 9410704) is available on request from Novo Nordisk A/S. The assay was performed without magnetic stirring, but in a shaking water bath.

EXAMPLE 2

65 g of Celkate T-21 was introduced into a high speed mixer as indicated in Example 1. 25 g of *Humicola lanuginosa* lipase liquid concentrate as 10 indicated in Example 1 was continuously atomized onto the powder with running impeller. Hereafter, 50 g of the *Humicola lanuginosa* lipase liquid concentrate with 3% (w/w) Kollidon K25 polyvinylpyrrolidone (BASF) was atomized onto the powder. The formed granulate was dried overnight at room temperature and sieved (300 - 700 μm). The moisture content was adjusted to 10% and the sample analyzed to 0.5 15 BAUN/g.

EXAMPLE 3

40 g of a powder of a calcinated diatomaceous earth, Clarcel CBL 3 (Ceca S.A.) was introduced into a high speed mixer as indicated in Example 1. 11 9 g of *Humicola lanuginosa* lipase liquid concentrate as indicated in Example 1 was 20 continuously atomized onto the powder with running impeller. Hereafter, 47 g of the *Humicola lanuginosa* lipase with 3% (w/w) Kollidon K25 was atomized onto the powder with running impeller. The formed granulate was dried overnight at room temperature and sieved (300 - 700 μm). The moisture content was adjusted to 10% and the sample analyzed to 2.4 BAUN/g.

EXAMPLE 4

50 g of Clarcel CBL 3 was introduced into a high speed mixer as indicated in Example 1. 72 g of *Humicola lanuginosa* lipase liquid concentrate as indicated in Example 1 with 5% (w/w) gelatin (ASF gelatin, Sanofi Bio-Industries) was 5 continuously atomized onto the powder liquid concentrate as indicated in Example 1. The formed granulate was dried overnight at room temperature and sieved (300 - 700 μ m). The moisture content was adjusted to 10% and the sample analyzed to 5.1 BAUN/g.

EXAMPLE 5

10 30 g of Clarcel CBL 3 and 20 g of talc powder was introduced into a high speed mixer as indicated in Example 1. 20 g of *Humicola lanuginosa* lipase liquid concentrate as indicated in Example 1 was continuously atomized onto the powder liquid concentrate as indicated in Example 1. Hereafter, 28 g of the *Humicola lanuginosa* lipase concentrate with 2% (w/w) Methocel A-15 15 methylcellulose (Dow) was atomized onto the powder with running impeller. The formed granulate was dried overnight at room temperature and sieved (300 - 700 μ m). The moisture content was adjusted to 10% and the sample analyzed to 7.7 BAUN/g.

EXAMPLE 6

20 250 g of Celkate T-21 was washed with 3 volumes of 0.1 M acetate buffer, pH 4.5, for 30 minutes, followed by vacuum filtration. *Humicola lanuginosa* lipase concentrate as indicated in Example 1 in an amount corresponding to 500,000 LU/g of Celkate T-21 was added together with 3 volumes of 0.1 M acetate buffer, pH 4.5, and stirred for two hours at room temperature. After vacuum filtration the 25 immobilized lipase was dried for 24 hours at room temperature, the moisture content

adjusted to 10% and analyzed to 14.3 BAUN/g. The filtrate contained 27565 kLU, corresponding to an adsorption of 78% (or 390 kLU/g).

65 g of the thus dried immobilized lipase on Celkate T-21 powder was introduced into a high speed mixer as indicated in Example 1. 55 g of a 5% (w/w) 5 gelatin solution was atomized onto the powder with running impeller. Hereafter, 0.1 g of Aerosil 200 silicium dioxide (Degussa) was added. The formed granulate was dried at room temperature and sieved (300 - 700 μ m). The moisture content was adjusted to 10% and analyzed to 5.9 BAUN/g.

EXAMPLE 7

10 200 g of Clarcel CBL 3 was washed with 3 volumes of 0.1 M acetate buffer, pH 4.5, for 30 minutes, followed by vacuum filtration. *Humicola lanuginosa* lipase concentrate as indicated in Example 1 in an amount corresponding to 500,000 LU/g of Clarcel CBL 3 was added together with 3 volumes of 0.1 M acetate buffer, pH 4.5, and stirred for two hours at room temperature. After vacuum filtration the 15 immobilized lipase was washed two times with 2 - 3 volumes of 0.1 M acetate buffer, pH 4.5, and two times with deionized water. The filtrates contained 82761 kLU in total corresponding to an adsorption of 17% (or 86 kLU/g). The immobilized lipase was dried for 24 hours at room temperature and analyzed to 13.4 BAUN/g.

55 g of the thus washed immobilized lipase on Clarcel CBL 3 powder 20 was introduced into a high speed mixer as indicated in Example 1. 61 g of a solution containing 2% (w/w) gelatin and 1% (w/w) Methocel A-15 methylcellulose (Dow) was continuously atomized onto the powder with running impeller. Hereafter, 0.1 g of Aerosil 200 silicium dioxide (Degussa) was added. The formed granulate was dried at room temperature and sieved (300 - 700 μ m). The moisture content was adjusted 25 to 10% and analyzed to 8.4 BAUN/g.

Another portion, i.e. 59 g of the thus washed immobilized lipase on Clarcel CBL 3 powder was introduced into a high speed mixer as indicated in Example 1. 59 g of a 5% (w/w) gelatin solution was continuously atomized onto the powder with running impeller. Hereafter, 0.1 g of Aerosil 200 silicium dioxide

(Degussa) was added. The formed granulate was dried at room temperature and sieved (300 - 700 μm). The moisture content was adjusted to 10% and analyzed to 10.1 BAUN/g.

EXAMPLE 8

5 This is a manufacturing example as Examples 1 - 7, but with another lipase producing microorganism.

Preparation of Sample 1: 12.9 g of *Candida antarctica* B lipase freeze dried powder with an activity of 250,000 LU/g and 1.4 g of Kollidon K25 was dissolved in 51 ml of deionized water. 50 g of Celkate T-21 was introduced into a high speed mixer as 10 indicated in Example 1 and the above indicated solution of *Candida antarctica* B lipase was continuously atomized onto the powder with running impeller. The formed granulate was dried overnight at room temperature and sieved (300 - 1000 μm).

Preparation of Sample 2: 12.9 g of *Candida antarctica* B lipase and 0.86 g of Methocel A-15 was dissolved in 51 ml of deionized water. 50 g of Celkate T-21 was 15 introduced into a high speed mixer and the above solution was continuously atomized onto the powder with running impeller. The formed granulate was dried overnight at room temperature and sieved (300-1000 μm).

Preparation of Sample 3: 12.8 g of *Candida antarctica* B lipase and 0.81 g of Kollidon K25 was dissolved in 48 ml deionized water. 50 g of Celkate T-21 was 20 introduced into a high speed mixer and the above solution was continuously atomized onto the powder with running impeller. The formed granulate was dried overnight at room temperature and sieved (300 - 1000 μm).

EXAMPLE 9

This is a use example with the preparation according to Example 8, associated with Fig. 1.

The three samples described in Example 8 were evaluated in columns 5 by continuous synthesis of ethyl glucosid esters (EGE) by reacting ethyl glucosid (EG) with decanoic acid.

Reaction conditions:

Column dimensions: diameter = 1.5 cm; length = 20 cm

Sample size: 5.0 g

10 Substrate: Ethyl glucosid*) 4.98 kg

Decanoic acid 4.92 kg

Tertiary butanol 25% 3.30 kg

Temperature: 60°C

Flow: 30 g/h

15 Time: 162 hours

*) Synthesized by reacting ethanol and D-glucose in the presence of a cation exchanger

Samples were taken after 18, 44, 90, and 162 hours and the content of EGE and EG was measured on HPLC and % conversion calculated. The results 20 are shown in Fig. 1. Moreover, it was noticed that the physical stability of the samples was good.

CLAIMS

1. Method for production of an immobilized enzyme preparation, comprising an enzyme applicable for organic synthesis in a mainly organic medium devoid of free water, characterized by the fact that a liquid enzyme composition and a particulate silica carrier with a particle size below around 100 μm is used as materials to be introduced into a granulator or extruder, whereafter a granulation or an extrusion is carried out.
2. Method according to Claim 1, characterized by the fact that the enzyme is a lipase.
- 10 3. Method according to Claim 2, characterized by the fact that the lipase in the liquid lipase composition is a thermostable lipase.
4. Method according to Claims 2 - 3, characterized by the fact that the lipase in the liquid lipase composition is produced by cultivation of a microorganism containing a gene encoding for and expressing a lipase derived from a strain of 15 *Humicola* species, *Candida antarctica* or *Rhizomucor miehei*.
5. Method according to Claims 2 - 4, wherein the proportion between the amount of the liquid lipase composition and the weight of particulate silica carrier is at least 100,000 LU/g of carrier (dry weight).
6. Method according to Claims 1 - 5, characterized by the fact that the 20 silica has a purity of at least 50%, preferably at least 75%.
7. Method according to Claims 1 - 6, characterized by the fact that a granulator is used, preferably a high speed mixer or a mixer granulator.

8. Method according to Claim 1 - 7, characterized by the fact that a liquid composition of a binder, preferably gelatin or polyvinylpyrrolidone, is introduced by atomization into the granulator or extruder during the granulation or extrusion.
9. Method according to Claims 1 - 8, characterized by the fact that the granulation or extrusion is carried out for production of the immobilized enzyme preparation with a particle size distribution corresponding to an amount of at least 90% between 50 μm and 2,000 μm .
10. Use of the immobilized enzyme preparation prepared according to Claims 1 - 9, for the process catalyzed by the enzyme.
- 10 11. Use of the immobilized lipase preparation prepared according to Claims 2 - 9, for fat related processes.
12. Use according to Claim 11 for interesterification of fats, characterized by the fact that liquid fats or fatty mixtures, including free fatty acids or fatty acid esters, are contacted with the immobilized lipase preparation.
- 15 13. Use according to Claim 11 for synthesis of glycerides or other fatty acid esters, characterized by the fact that a mixture of glycerol or substituted glycerols or other types of alcohols and free fatty acids is contacted with the immobilized lipase preparation.
14. Use according to Claim 13, characterized by the fact that the fatty acid esters are glycolipids.

1/1

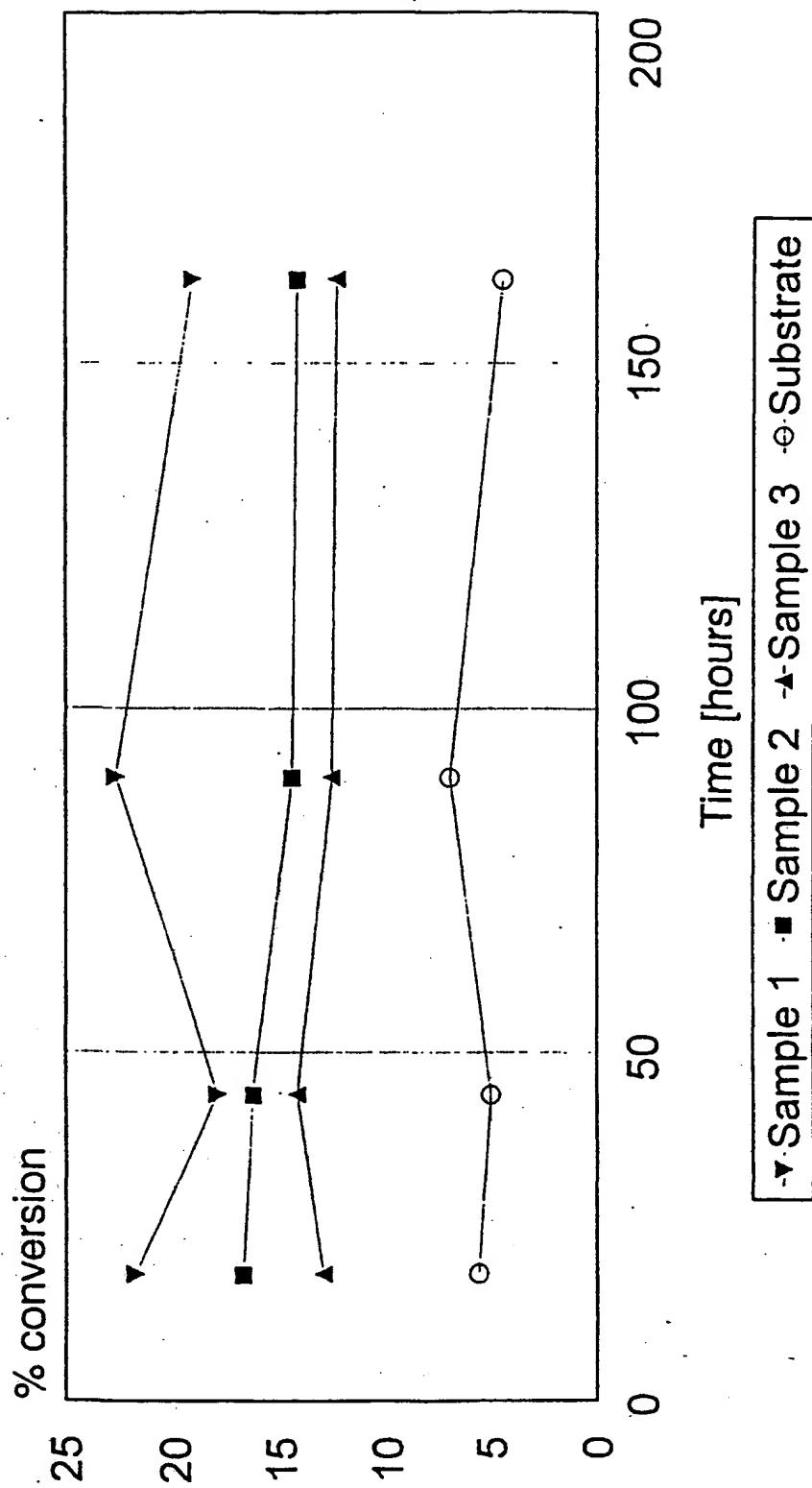


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00076

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 11/14, C12N 9/20 // C01B 33/20
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, DERWENTBIOCHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A2, 0159578 (REDCO N.V.), 30 October 1985 (30.10.85), page 9, line 1 - line 4 --	1-14
Y	WO, A1, 9005778 (NOVO-NORDISK A/S), 31 May 1990 (31.05.90) --	1-14
A	EP, A1, 0093027 (ARGILES & MINERAUX AGS-BMP), 2 November 1983 (02.11.83), claim 5 --	1-14

Further documents are listed in the continuation of Box C.

See patent family annex.

- * Special categories of cited documents
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" prior art document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

2 June 1995

Date of mailing of the international search report

12-06-1995

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. + 46 8 666 02 86

Authorized officer

Patrick Andersson
Telephone No. + 46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00076

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>WO, A2, 9426883 (GIST-BROCADES N.V.), 24 November 1994 (24.11.94), page 5, line 4 - line 8; page 6, line 5 - line 8</p> <p>---</p> <p>-----</p>	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/DK 95/00076

03/05/95

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0159578	30/10/85	SE-T3- EP-A,A,A SE-T3- JP-C- JP-B- JP-A- JP-A- US-A- US-A-	0159578 0158909 0158909 1805034 5013090 60232090 61006118 4683203 4689315	23/10/85 26/11/93 19/02/93 18/11/85 11/01/86 28/07/87 25/08/87
WO-A1- 9005778	31/05/90	CA-A- DE-T- DK-B- EP-A,B- JP-T- US-A-	2003163 68907611 169951 0444092 4501664 5342768	16/05/90 21/10/93 10/04/95 04/09/91 26/03/92 30/08/94
EP-A1- 0093027	02/11/83	FR-A,B- US-A- FR-A,A,B	2525629 4560660 2533229	28/10/83 24/12/85 23/03/84
WO-A2- 9426883	24/11/94	NONE		

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.